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Immunological detection of winter flounder (*Pseudopleuronectes americanus*) eggs and juveniles in the stomach contents of crustacean predators

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Abstract

Predation on the early life history of fish is an important factor regulating year-class strength. Verifying predation events, however, is difficult when analyses rely on visually identifying the remnants of partially digested fish in the stomachs of suspected predators. The objective of this study was to assess the utility of using immunological assays to detect the presence of winter flounder eggs and juveniles (*Pseudopleuronectes americanus*) in the gut contents of sand shrimp (*Crangon septemspinosa*) and green crab (*Carcinus maenas*). After defining assay capabilities, the stomach contents of field-collected shrimp and crabs were examined to determine if these predator–prey relationships occur under natural conditions. Winter flounder-specific antisera developed and used in this study successfully identified homologous antigens (egg or juvenile flounder extracts) without appreciably cross-reacting with antigenic material from predators or nontarget prey. Moreover, antisera detected flounder eggs 10.8–16.4 h after initial feeding by various sized shrimp, and identified juvenile flounder 9.4 and 7.8 h after initial ingestion by shrimp and crabs, respectively. Immuonological dietary analysis of decapod crustaceans collected from Niantic River, Connecticut, revealed that *C. septemspinosa* and *C. maenas* are potentially important predators on the early life stages of winter flounder. The temporal trends and magnitude of flounder predator-induced mortality was affected primarily by the spatial and temporal overlap between predator and prey (egg mortality), and the size-dependent relationships underlying crustacean and flatfish predator–prey interactions (juvenile mortality).

Keywords: *Carcinus maenas*; *Crangon septemspinosa*; Crustacean; Immunoassay; Predation; Stomach contents; Winter flounder

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1. Introduction

A fundamental objective of fisheries research is to understand the factors that influence year-class strength. There is a general consensus that the size of a given year-class is controlled during a critical period in the early life history of fish, when mortality rates are substantial and highly variable. Of the potential physical and biotic factors thought to regulate year-class formation, predation on the early life stages of fish is perhaps the most important (Bailey and Houde, 1989).

For several species of flatfish, year-class size is determined at the post-settlement stage and shortly thereafter (Edwards and Steele, 1968; Lockwood, 1980; Zijlstra et al., 1982; Van der Veer and Bergman, 1987). Predation by decapod crustaceans is a source of high mortality for newly settled flatfish and therefore a possible determinant of year-class strength. Among decapod crustaceans, crangonid shrimp (*Crangon* spp.) are the most recognized predators of juvenile flatfish (Van der Veer and Bergman, 1987; Seikai et al., 1993; Witting and Able, 1995; Yamashita et al., 1996). In the North Sea and western Wadden Sea, for example, brown shrimp (*Crangon crangon*) are important predators of juvenile plaice (*Pleuronectes platessa*) and flounder (*Platichthys flesus*) during and shortly after the fish settle on tidal flats (Van der Veer and Bergman, 1987). In Sendai Bay, Japan, the stomach contents of *C. affinis* were documented to contain otoliths of the stone flounder (*Kareius bicoloratus*) when the two species co-occur on flatfish nursery grounds (Yamashita et al., 1996). Several species of brachyuran crabs, including the green crab (*Carcinus maenas*), blue crab (*Callinectes sapidus*), and sandy shore crab (*Matuta lunaris*), also preyed on juvenile flatfish during laboratory feeding experiments (Van der Veer and Bergman, 1987; Fairchild and Howell, 2000; Kellison et al., 2000; Hossain et al., 2002).

The winter flounder (*Pseudopleuronectes americanus*) is a pleuronectid flatfish inhabiting temperate estuaries of the northwest and mid-Atlantic (Scott and Scott, 1988). The unique life cycle of winter flounder makes this species particularly vulnerable to epibenthic predators during two early life stages—the egg and juvenile period. Spawning of winter flounder eggs occurs inside estuaries in winter and early spring at temperatures ranging from 1 to 10 °C, with peak spawning at 2–5 °C (Pearcy, 1962). The eggs are adhesive and demersal, with incubation lasting 5–31 days (Rogers, 1976). During incubation, clusters of winter flounder eggs could experience substantial mortality due to epibenthic predation. The highly aggregated distribution of fish eggs on spawning grounds can attract high densities of predatory fish, diving birds, and invertebrates (Taylor, 1964; Frank and Leggett, 1984; Haegele, 1993a,b). Haegele (1993b) examined invertebrate predation on Pacific herring eggs (*Clupea pallasi*) and estimated that on average 3.7% of a given herring spawn was consumed, with decapod crustaceans accounting for the majority of the total eggs eaten (60%). Moreover, Taylor (in preparation) observed that sand shrimp (*Crangon septemspinosa*) and green crabs (*C. maenas*) readily consumed aggregations of winter flounder eggs under laboratory conditions.

After winter flounder eggs hatch, the pelagic larval stage lasts approximately 60 days and concludes with the metamorphosis and settlement of juveniles to the benthos (Pearcy, 1962). The small size of recently settled winter flounder (8 to 9 mm total length, TL; Chambers and Leggett, 1987) and their relatively weak swimming and burying capabilities

renders the flatfish susceptible to crustacean predators. Laboratory feeding experiments, for example, have implicated *C. septemspinosa* and *C. maenas* as key predators of newly metamorphosed winter flounder (Witting and Able, 1995; Fairchild and Howell, 2000; Taylor, in press).

Despite laboratory evidence suggesting that crustaceans are potentially important predators of winter flounder eggs and post-settlement juveniles, to date there is no direct evidence that these predator–prey relationships exist in the field. Visual estimates of a predator's stomach contents have traditionally been used to verify trophic linkages under field conditions. For example, previous attempts to identify winter flounder in the diet of shrimp and crabs have relied on the visual identification of flounder remains, including fish otoliths, scales, and fin rays, i.e., hard parts that are not easily digested by the predator. However, visually identifying flounder in crustacean diets is hampered due to: (1) the mastication of flounder tissue by mandible and gastric mill grinding, (2) the ingestion of particulate matter and other prey items that visually resemble flounder hard parts, and (3) partial or incomplete predation events that do not result in the ingestion of identifiable flounder remains. Visual analyses are therefore extremely difficult, time-consuming, and may lead to erroneous conclusions.

As an alternative approach to visual estimations, biochemical techniques provide a means of positively identifying prey proteins in a predator's stomach. Immunological assays, for example, use the highly specific binding and recognition capabilities of antibodies to identify immunogenic moieties (antigens) present in the stomach contents of predators (Feller, 1991). Previous studies have successfully used immunological techniques to identify the stomach contents of several marine crustaceans, including penaeid shrimp (Hunter and Feller, 1987; McTigue and Feller, 1989; Hentschel and Feller, 1990), crangonid shrimp (*C. crangon*) (Van der Veer et al., 1998), green crab (*C. maenas*) (Van der Veer et al., 1998), and a variety of other benthic invertebrates (Feller et al., 1979). Moreover, immunological studies have assessed the predation mortality of juvenile red drum (*Sciaenops ocellatus*) by bar jacks (*Caranx ruber*) (Schultz and Clarke, 1995), and also crustacean consumption of eggs and yolk-sac larvae of walleye pollock (*Theragra chalcogramma*) (Bailey et al., 1993) and northern anchovy (*Engraulis mordax*) (Theilacker et al., 1986, 1993).

The objective of this study was to assess the utility of using immunological assays to detect the presence of winter flounder eggs in the stomach contents of the sand shrimp (*C. septemspinosa*), and post-settlement juveniles in the stomachs of *C. septemspinosa* and green crab (*C. maenas*). After defining the immunological capabilities, the stomach contents of field-collected predators were examined to determine if these predator–prey relationships occur under natural conditions.

2. Materials and methods

Preliminary visual analysis of the stomach contents of field-collected *C. septemspinosa* and *C. maenas* revealed only discolored amorphous material, left unidentifiable following crustacean mandible and gastric mill grinding. As an alternative approach, the Öuchterlony double-diffusion immunoassay was used to identify the presence or absence of winter

flounder proteins in the stomach contents of suspected shrimp and crab predators. Before dietary analysis of field-collected shrimp and crabs, several initial procedures were performed to ensure that the given predator–prey relationships could be examined effectively with immunoassays: (1) preparation of antigen and antibody, (2) testing of antibody sensitivity and specificity, and (3) determination of detection limits.

2.1. Antigen preparation

Winter flounder eggs and juveniles were obtained and raised from broodstock that were collected by otter trawl from Narragansett Bay (Rhode Island, USA). Adult flounder constituting the broodstock were transferred and maintained in laboratory tanks supplied with running seawater at ambient temperature and salinity (4–6 °C and 28–30 ppt). The collection and fertilization of winter flounder eggs and rearing of juveniles was performed using techniques described by Klein-MacPhee et al. (1982). Whole-animal extracts were prepared from fertilized flounder eggs that were 2–10 days post-spawn and juvenile flounder that measured between 8 and 30 mm total length (TL). Prior to antigen preparation, juvenile flounder were starved for 48 h to allow the complete evacuation of stomach contents, after which samples of both juveniles and eggs were rinsed in distilled water, blotted dry, and frozen at −20 °C. Upon antigen preparation, 1.0 g of frozen egg or juvenile tissue was ground with 0.25 ml of buffer solution (TES–saline: 5 mM TES [*N*-tris(hydroxtmethyl)methyl-2-aminoethane sulfonic acid], 30 mM NaOH, and 150 mM NaCl, pH = 7.3). Grinding was performed with a motorized pellet pestle in an ice bath to prevent thermal denaturation of proteins. After the tissue samples were solubilized in TES–saline, the suspension was centrifuged (Eppendorf Centrifuge 5417C) for 12 min at 7400 × *g*, and the supernatant was withdrawn and stored by freezing at −20 °C. A total of 10 ml of winter flounder egg and juvenile antigen solutions were prepared. Protein concentrations of the extracts were measured spectrophotometrically (Cambridge Instruments 7520 Microplate Reader) using the protocol of Smith et al. (1985) adapted for a 96-well microplate format. Samples and standards plus reagents were incubated at 60 °C for 1 h to allow for complete color development. A serial dilution of bovine serum albumin was used as a standard. The protein concentration of winter flounder egg and juvenile extracts was 44.8 and 14.3 mg ml^{-1}, respectively.

2.2. Antibody preparation

Polyclonal antibody preparation was performed using techniques described by Feller et al. (1979) with minor modifications. Extracts (winter flounder egg and juvenile antigen solutions) were injected into pairs of New Zealand white female rabbits (approximately 3.0 kg body weight) that had no prior immunization history. Approximately 10 ml of whole blood was collected from the central ear artery of each rabbit 1-week prior to the start of the immunization sequence. Pre-immunization blood was allowed to clot at 5 °C for 24 h, after which the serum was collected and stored at −20 °C. The pre-immunization serum was tested against egg and juvenile flounder extracts to check that no antibodies to the extracts were present in rabbits before immunization.

Flounder egg and juvenile extracts were emulsified in an equal volume of Freund's incomplete adjuvant (Difco). A mixture of 1 ml of egg or juvenile extract plus 1 ml of adjuvant was injected intramuscularly into the rabbit's flank on day 1 of the immunization schedule. On day 21, each rabbit was injected with 0.1 ml of either egg or juvenile full-strength extract (without adjuvant) intravenously via a marginal ear vein. On day 24, 0.2 ml; on day 27, 0.3 ml; and on day 30, 0.4 ml of full-strength extract was administered in the same manner. On day 37, the rabbits were anesthetized and bled by cardiac puncture. Approximately 150 ml of whole blood was collected from each rabbit and was allowed to clot at 5 °C for 24 h, after which the serum was withdrawn and stored at −20 °C. Each rabbit produced approximately 75 ml of serum (polyclonal antibody solution).

2.3. Sensitivity and specificity testing

The ability of antibodies to detect target prey (i.e., sensitivity or self-reactions) was determined by testing post-immunization antisera against the same (homologous) extracts from which the antisera were prepared. Qualitative immunoassays, utilizing the micro-Öuchterlony double-diffusion technique (Feller et al., 1979), were performed by placing two plastic templates (25 × 25 mm) on an agarose matrix that covered a standard glass microscope slide (25 × 75 mm). Each template consisted of five wells, into which 20 μl of egg or juvenile antisera were pipetted into a single central well. The remaining four additional wells surrounding and equidistant from the template's central well were loaded with 20 μl of antigen solutions prepared from whole-animal extracts of flounder eggs or juveniles (or solubilized gut material from suspected predators: see Sections 2.4 and 2.5). The antisera drained through a small hole (diameter = 1 mm) at the bottom of the central well and diffused radially through the agarose until coming in contact with the antigen solutions diffusing towards the central well. The diffusion process occurred over a 48-h period in a humidified chamber, after which templates were removed and the slides were immersed in a TES–saline bath for 24 h to rid the agarose gel of unprecipitated proteins. The slides were then washed in distilled water for 4 h to remove salts, dried at 37 °C overnight, and stained with Coomassie brilliant blue R (protein stain) to highlight the precipitin lines. Precipitin lines form between the central well and the peripheral wells if antibodies within the antiserum react with the soluble antigenic proteins. The strength of antibody–antigen self-reactions was quantified by counting the number of precipitin lines formed between template wells. Four independent assays were performed for each sensitivity test ($n = 4$).

Post-immunization antisera were tested for specificity by determining cross-reactions with the gut and muscle tissue of the predators *C. septemspinosa* and *C. maenas*, as well as cross-reactions with possible alternative (nontarget) prey of shrimp and crabs (Table 1). Animal extracts (heterologous antigen preparation) of *C. septemspinosa*, *C. maenas*, and nontarget prey were performed using the techniques described above, and specificity was examined with immunoassays in the same manner as sensitivity testing. The strength of cross-reactions was quantified by counting the number of precipitin lines formed between template wells. Four independent assays were performed for each specificity test ($n = 4$).

Table 1
Winter flounder (*P. americanus*) egg and juvenile antisera self-reactions with homologous antigens (bold text) and cross-reactions with heterologous antigens (normal text)[a]

Phylum	Species	Common name	Antisera	
			Egg	Juvenile
Chordata	*Pseudopleuronectes americanus* (egg)	Winter flounder	**14.8 ± 0.3**	3.5 ± 0.3
	Pseudopleuronectes americanus (juvenile)	Winter flounder	4.8 ± 0.3	**13.8 ± 0.8**
	Pseudopleuronectes americanus (larvae)	Winter flounder	1.8 ± 0.5	2.3 ± 0.3
	Brevoortia tyrannus	Atlantic menhaden	1.0 ± 0.0	1.3 ± 0.3
	Fundulus majalis	Striped killifish	1.5 ± 0.3	2.3 ± 0.3
	Gasterosteus aculeatus	Threespine stickleback	1.5 ± 0.3	2.5 ± 0.3
	Gobiosoma bosci	Naked goby	2.0 ± 0.0	2.0 ± 0.0
	Menidia menidia	Atlantic silverside	2.5 ± 0.3	3.0 ± 0.4
	Menticirrhus saxatilis	Northern kingfish	2.8 ± 0.3	4.0 ± 0.4
	Myoxocephalus scorpius	Shorthorn sculpin	0.5 ± 0.3	2.5 ± 0.5
	Syngnathus fuscus	Northern pipefish	1.3 ± 0.3	2.5 ± 0.3
	Tautoga onitis	Tautog	2.0 ± 0.4	3.0 ± 0.0
	Tautogolabrus adspersus	Cunner	1.3 ± 0.3	2.0 ± 0.0
Arthropoda	*Carcinus maenas* (muscle)	Green crab	0.0 ± 0.0	0.0 ± 0.0
	Carcinus maenas (gut)	Green crab	0.0 ± 0.0	0.3 ± 0.3
	Crangon septemspinosa (muscle)	Sand shrimp	0.0 ± 0.0	0.0 ± 0.0
	Crangon septemspinosa (gut)	Sand shrimp	0.0 ± 0.0	0.0 ± 0.0
	Crangon septemspinosa (egg)	Sand shrimp	0.3 ± 0.3	0.0 ± 0.0
	Gammarus oceanicus	Amphipod	0.5 ± 0.3	0.3 ± 0.3
	Idotea baltica	Isopod	0.8 ± 0.3	0.0 ± 0.0
	Neomysis americana	Mysid shrimp	0.0 ± 0.0	0.0 ± 0.0
	Pagurus longicarpus	Long-clawed hermit crab	0.3 ± 0.3	0.0 ± 0.0
	Palaemonetes sp.	Grass shrimp	0.0 ± 0.0	0.0 ± 0.0
Annelida	*Glycera* sp.	Blood worm	0.3 ± 0.3	0.0 ± 0.0
	Neries sp.	Clam worm	0.0 ± 0.0	0.0 ± 0.0
Mollusca	*Mya arenaria*	Soft-shell clam	0.0 ± 0.0	0.0 ± 0.0
	Mulinia sp.	Infaunal clam	0.0 ± 0.0	0.0 ± 0.0
	Yoldia limulata	Infaunal clam	0.0 ± 0.0	0.0 ± 0.0

[a] Values represent the mean number of precipitin lines (± 1 S.E.) formed during antibody–antigen reactions. Four independent assays were performed for each test ($n = 4$).

Differences in the number of precipitin lines formed when antisera were tested against homologous or heterologous antigens (self- and cross-reactions, respectively) were analyzed with a one-way analysis of variance (ANOVA) model using species-specific (and life stage-specific for winter flounder) antigen as a fixed factor (Table 1). Mean numbers of precipitin lines created between antisera and 28 different antigens were contrasted with Tukey's multiple comparison test. Differences in the strength of cross-reactions (number of precipitin lines) grouped by phyla were also analyzed with a one-way ANOVA model using phyla as a fixed factor. Mean numbers of precipitin lines across four levels of phyla [Chordata (excluding winter flounder), Arthropoda, Annelida, Mollusca] were contrasted with Ryan–Einot–Gabriel–Welsch (Ryan's Q) multiple comparison test. Ryan's Q multiple comparison test was used in cases of unequal sample sizes (phyla contrasts), as opposed to Tukey's multiple comparison test, which was used in situations with equal sample sizes (species contrasts) (as recommended by Day and Quinn, 1989).

2.4. Detection limits

The effect of digestion time on detecting flounder proteins in crustacean stomachs was determined in laboratory feeding experiments. *C. septemspinosa* and *C. maenas* were collected by otter trawl (February to March; shrimp only) and beam trawl (June to July; shrimp and crabs) from Niantic River (Connecticut, USA), transferred to the laboratory, and maintained in large flow-through tanks (4–18 °C and 28–30 ppt). The detection of juvenile flounder in predator stomachs was restricted to shrimp measuring 45–65 mm total length (TL) and crabs measuring 49–61 mm carapace width (CW). Shrimp and crabs of these sizes prey on post-settlement winter flounder under laboratory conditions (Witting and Able, 1995; Fairchild and Howell, 2000). Conversely, the body size of *C. septemspinosa* does not constrain shrimp from feeding on winter flounder eggs (Taylor, in preparation). Accordingly, detection of eggs in the stomach contents of *C. septemspinosa* was examined over a wide range in shrimp size (small = 20–30 mm TL, medium = 31–45 mm TL, large = 46–65 mm TL).

Shrimp and crabs were starved 48 h to ensure that all previous stomach contents were evacuated prior to the initiation of feeding experiments. Feeding experiments were conducted at temperatures that reflected conditions experienced by the specific life stages of winter flounder in the field. Accordingly, shrimp maintained in 39-l aquaria at 6.0 ± 0.2 °C were fed flounder eggs (0.01–0.05 g wet weight), and shrimp and crabs maintained at 13.0 ± 0.2 °C were fed juvenile flounder tissue (0.05–0.10 g wet weight). The stomach contents of predators were collected at several time points after initial feeding (0–6 h post-feeding), solubilized in TES–saline buffer solution, centrifuged to remove particulates, and the supernatant was assayed as previously described. Differences in detection responses (number of precipitin lines) were analyzed by an analysis of covariance (ANCOVA) model with time after initial feeding (0–6 h post-feeding) as the covariate and shrimp size (small, medium, or large) or predator type (shrimp or crab) as the discrete explanatory variable.

2.5. Analysis of field-collected predators

Immunoassays were performed on field-collected *C. septemspinosa* and *C. maenas* after defining the capability of antisera to detect target prey in predator stomachs from known samples. Shrimp (22–65 mm TL; $n = 600$) and adult winter flounder were collected by otter trawl from 18 February to 1 April 2002 in Niantic River, Connecticut. The reproductive condition (gravid or not gravid) of adult female flounder (>26 cm TL) collected in trawls was documented and used as an approximation of flounder spawning period and the relative abundance of spawned eggs. Collected shrimp were placed on ice for approximately 1 h and then frozen at − 20 °C once returning to the laboratory. Upon gut content analysis, shrimp were thawed and stomach contents were prepared and assayed as described. The antigenic material in the shrimp's stomach was tested against flounder egg antiserum, and a positive result was defined as the formation of at least one distinct precipitin line. The resulting reaction was then confirmed with tests of identity of the antigenic material in gut content samples and the native antigenic material used to create the antiserum (Hunter and Feller, 1987; Feller, 1991). Logistic regression analysis,

employing the method of maximum likelihood, was used to test for a significant relationship between egg predation (response frequencies: presence or absence of eggs in shrimp guts) and sampling date (day 49 to day 90).

Shrimp (36–64 mm TL; $n = 255$), crabs (14–71 mm CW; $n = 138$), and post-settlement flounder were collected by beam trawl from 24 May to 1 August 2001 in Niantic River, Connecticut. Field sampling was limited to nearshore, shallow areas that were inhabited by shrimp, crabs, and juvenile flounder during late spring and summer. Juvenile flounder collected in trawls were measured to the nearest millimeter and returned to the estuary. Suspected shrimp and crab predators were placed on ice for approximately 1 h and then frozen at − 20 °C once returning to the laboratory. Upon gut content analysis, predator stomach samples were prepared and analyzed with assays as described, with the exception that gut antigenic material was tested against juvenile antiserum to determine the incidence of predation on post-settlement flounder. Positive recognition of juvenile flounder in stomach samples was defined as the formation of at least one distinct precipitin line that was confirmed by tests of identity. Logistic regression was used to test for significant relationships between predation on juvenile flounder (response frequencies: presence or absence of juveniles in predator guts), sampling date (day 144 to day 212), and predator type (shrimp or crab).

3. Results

3.1. Sensitivity and specificity testing

Winter flounder antisera were tested for: (1) self-reactions with egg and juvenile homologous extracts, and (2) cross-reactions with antigens from alternative life stages of flounder and heterologous antigens from 22 different species representing four phyla (Table 1). Flounder egg antiserum reacted more strongly with its homologous egg antigen producing an average of 14.8 precipitin lines (Tables 1 and 2; Tukey's multiple

Table 2
Summary statistics for analysis of variance of the effects of species antigens (homologous and heterologous extracts) and phyla antigens (heterologous extracts) on the number of precipitin lines formed when reacted with winter flounder (*P. americanus*) egg and juvenile antisera

Source	SS	MS	(*df*)	*F*	*p*
Egg antiserum					
Species	876.61	32.47	(27)	165.29	< 0.0001
Error	16.50	0.20	(84)		
Phyla	53.69	17.90	(3)	53.64	< 0.0001
Error	32.03	0.33	(96)		
Juvenile antiserum					
Species	806.21	29.86	(27)	122.35	< 0.0001
Error	20.50	0.24	(84)		
Phyla	146.06	48.69	(3)	146.52	< 0.0001
Error	31.90	0.33	(96)		

comparison test). Egg antiserum produced significantly fewer lines when reacted with larval and juvenile flounder extracts (1.8 and 4.8 lines, respectively) and nontarget prey (average = 0.8 lines) (Tables 1 and 2; Tukey's multiple comparison test). Cross-reactions varied significantly according to phyla, whereby the strength of response was greater for Chordata (1.6 lines), as compared to Arthropoda (0.2 lines), Annelida (0.2 lines), and Mollusca (0.0 lines) (Tables 1 and 2; Ryan's multiple comparison test).

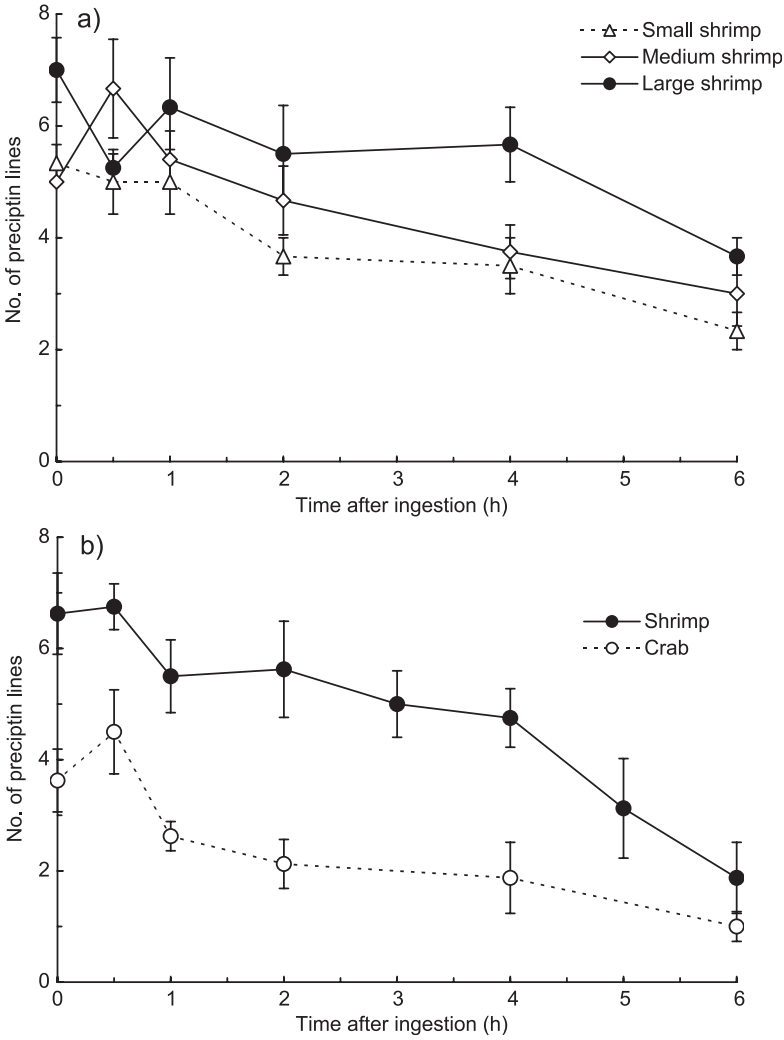

Fig. 1. Detection response (number of precipitin lines) of winter flounder (*P. americanus*) eggs (a) and juveniles (b) in the stomach contents of *C. septemspinosa* and *C. maenas* as a function of time after initial ingestion (h). Mean numbers of precipitin lines (± 1 S.E.) at individual time points after feeding are plotted ($n = 3–6$ for egg detection and $n = 8$ for juvenile detection at each time point).

Juvenile flounder antiserum reacted more strongly with its homologous antigen producing an average of 13.8 precipitin lines (Tables 1 and 2; Tukey's multiple comparison test). Cross-reacting juvenile antiserum with extracts of flounder egg and larval life stages resulted in significantly fewer lines (3.5 and 2.3 lines, respectively) (Tables 1 and 2; Tukey's multiple comparison test). Cross-reactions were also significantly weaker for nontarget prey (average = 1.2 lines) and varied according to phyla, whereby the strength of response was greater for Chordata (2.5 lines), as compared to Arthropoda (0.1 lines), Annelida (0.0 lines), and Mollusca (0.0 lines) (Tables 1 and 2; Ryan's multiple comparison test). In summary, the polyclonal antibodies used in this study have high sensitivity and high specificity, evident by flounder-specific antisera detecting the soluble proteins for which they were intended with minimal cross-reactions with predators and nontarget prey.

3.2. Detection limits

Winter flounder egg antiserum successfully recognized target prey in the stomach contents of shrimp (Fig. 1a). However, the strength of egg detection after initial feeding decreased by 53–66% relative to reactions observed with pure homologous egg extracts (Table 1; Fig. 1a). Prior to analyzing the effect of digestion time on egg detection, the interaction between shrimp body size and time after initial feeding was statistically examined. Accordingly, there was no significant interaction effect between size and time after feeding (Table 3), and the assumption of equal slopes was met in the full data set (Underwood, 1981). The strength of egg detection in shrimp stomachs increased with increasing predator size, whereby the average number of precipitin lines formed during the first 6 h after feeding was 5.6 lines for large shrimp, 4.7 lines for medium shrimp, and 4.1 lines for small shrimp (Fig. 1a). The difference in mean number of precipitin lines as a function of shrimp size, however, was not significant at the $p < 0.05$ level (Table 3). The strength of egg detection significantly decreased with time at a comparable rate among shrimp sizes and ranged between 40% and 56% from initial ingestion to 6 h post-feeding (Fig. 1a; Table 3). Assuming that detection levels decreased at the same rate observed for

Table 3
Summary statistics for analysis of covariance of the effect of predator digestion time on detection response of winter flounder (*P. americanus*) eggs and juveniles in the stomach contents of *C. septemspinosa* and *C. maenas*

Source	SS	MS	(*df*)	*F*	*p*
Egg detection					
Size	6.57	3.28	(2)	2.64	0.0796
Time	52.81	52.81	(1)	42.54	< 0.0001
Size × time	0.54	0.27	(2)	0.22	0.8046
Error	72.01	1.24	(58)		
Juvenile detection					
Predator type	115.87	115.87	(1)	38.09	< 0.0001
Time	174.10	174.10	(1)	57.23	< 0.0001
Predator type × time	7.74	7.74	(1)	2.55	0.1135
Error	328.53	3.04	(108)		

the first 6 h after ingestion, the detection limit (at least one precipitin line) for flounder eggs in shrimp stomachs is estimated to be 16.4 h for large shrimp, 12.1 h for medium shrimp, and 10.8 h for small shrimp.

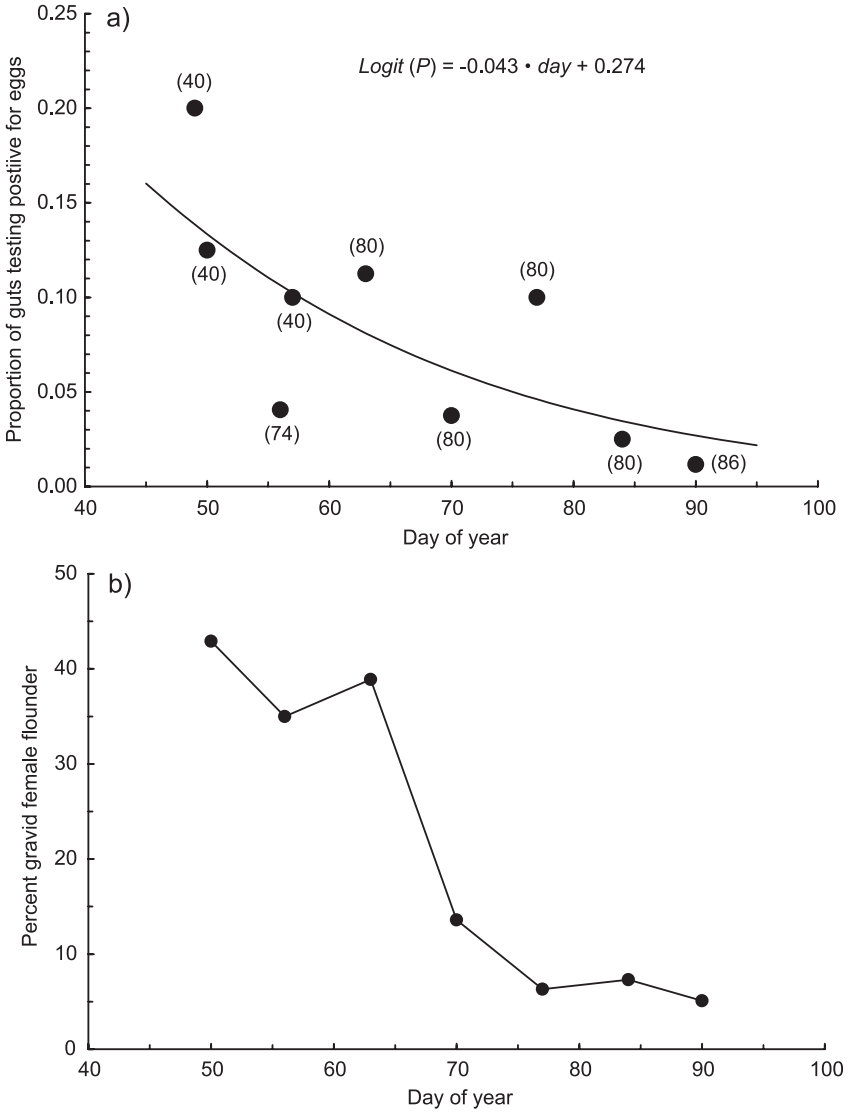

Fig. 2. Proportion of *C. septemspinosa* stomach contents testing positive for winter flounder (*P. americanus*) eggs (a). Values in parentheses represent sample size. Logistic regression estimated from maximum likelihood analysis was fit to data and the equation is presented: logit $(P) = \log[P/(1-P)]$, where P is proportion of shrimp guts testing positive for target prey. Percentage of gravid female winter flounder larger than 26 cm (b). Shrimp and adult flounder were collected in Niantic River, Connecticut, from 18 February to 1 April 2002.

Juvenile flounder antiserum recognized homologous antigens present in the stomach contents of shrimp and crabs (Fig. 1b). Similar to the detection of flounder eggs in shrimp stomach samples, juvenile antiserum recognition of digested flounder tissue decreased approximately 52% for shrimp and 74% for crabs after initial feeding (Table 1; Fig. 1b). There was no significant interaction effect between predator type (shrimp or crab) and digestion time (Table 3); hence permitting the use of an ANCOVA model (Underwood, 1981). The strength of juvenile detection was significantly greater for shrimp as compared to crabs, whereby the average number of precipitin lines formed during the first 6 h after feeding was 4.9 lines for shrimp and 2.6 lines for crabs (Fig. 1b; Table 3). For both predator types detection levels significantly decreased with time such that the capability of recognizing juvenile flounder in shrimp and crab stomachs decreased by 72% by 6 h after initial ingestion (Fig. 1b; Table 3). The detection limit of juvenile flounder in predator stomachs is projected to occur at 9.4 h post-feeding for shrimp and 7.8 h post-feeding for crabs.

3.3. Analysis of field-collected predators

Six hundred *C. septemspinosa* were collected from winter flounder spawning sites (Feb to Apr), of which 43 of the shrimp stomachs examined tested positive for flounder eggs (7.2%). The incidence of eggs in shrimp guts was greatest in mid-February (20.0%) and significantly decreased at a decelerating rate to 1.2% by early April (Fig. 2a; Table 4). The temporal trend in the proportion of shrimp guts containing eggs corresponded to a decrease in gravid female winter flounder collected during the observation period (Fig. 2b).

Table 4
Summary statistics for logistic regression analysis of presence or absence of winter flounder (*P. americanus*) egg and juveniles in the stomach contents of *C. septemspinosa* and *C. maenas* collected from Niantic River, Connecticut

Source	Parameter estimate (S.E.)	χ^2	*P*
Egg predation			
Intercept	0.274 (0.834)	0.11	0.7422
Date	− 0.043 (0.013)	10.92	< 0.005
Juvenile predation (combined)			
Intercept	1.246 (2.678)	0.22	0.6417
Predator type	− 7.656 (2.678)	8.17	< 0.005
Date	− 0.028 (0.017)	2.83	0.0927
Predator type × date	0.046 (0.017)	7.61	< 0.01
Juvenile predation (shrimp)			
Intercept	8.902 (4.208)	4.48	< 0.05
Date	− 0.074 (0.028)	7.06	< 0.01
Juvenile predation (crab)			
Intercept	− 6.410 (3.312)	3.74	0.0530
Date	0.018 (0.018)	0.96	0.3266

Accordingly, the percentage of gravid flounder was maximal in mid-February (42.9%) and decreased substantially to 5.1% by early April, marking the end of the winter flounder spawning season.

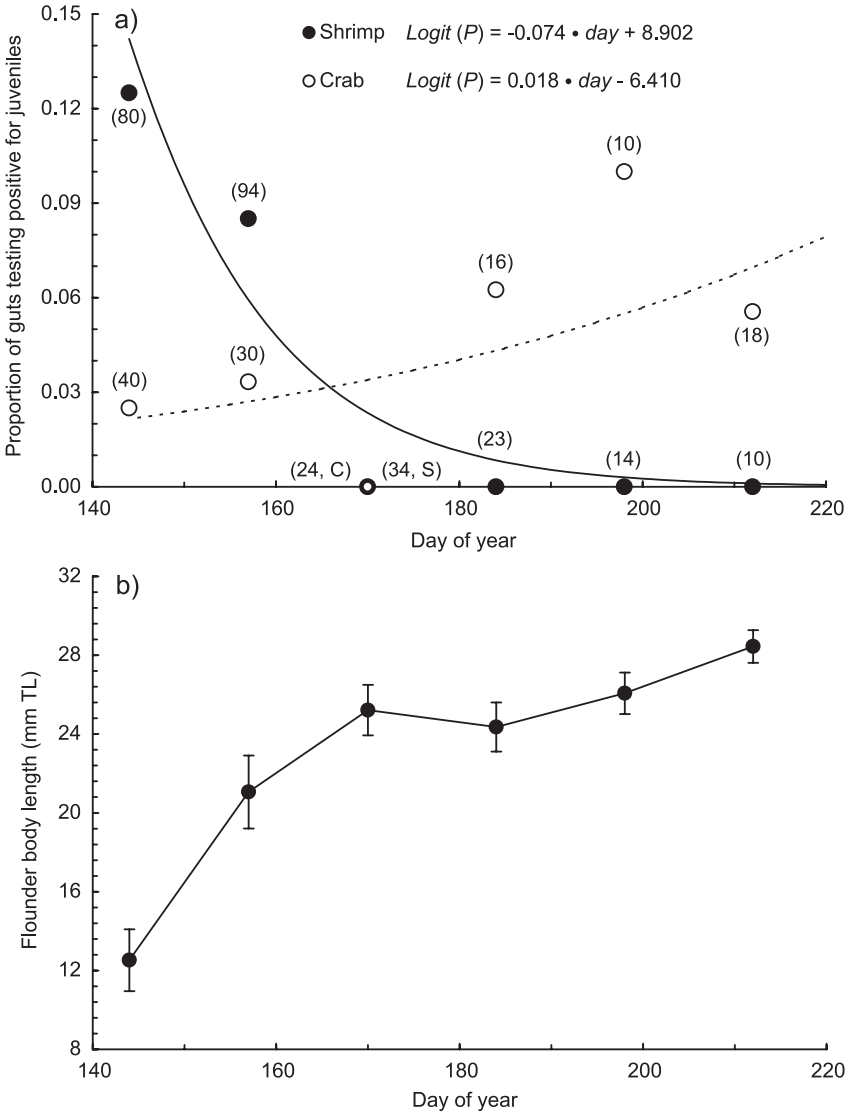

Fig. 3. Proportion of *C. septemspinosa* and *C. maenas* stomach contents testing positive for juvenile winter flounder (*P. americanus*) (a). Values in parentheses represent sample size. Logistic regressions estimated from maximum likelihood analysis were fit to data (solid line = shrimp and dashed line = crab) and the equations are presented: logit (P) = log[$P/(1-P)$], where P is proportion of predator guts testing positive for target prey. Mean total length (mm) of post-settlement flounder (± 1 S.E.) (b). Suspected predators and juvenile flounder were collected in Niantic River, Connecticut, from 24 May to 1 August 2001.

C. septemspinosa were also collected from flounder nursery habitats during late spring and summer. Of the 255 shrimp analyzed, 18 tested positive for juvenile flounder (3.5%). The percentage of shrimp stomachs testing positive was greatest in late May (12.5%) and early June (8.5%) (Fig. 3a). This percentage then decreased significantly and remained at 0.0% for the duration of summer field sampling (Fig. 3a; Table 4). The stomach contents of *C. maenas* also tested positive for juvenile flounder in late spring and early summer, albeit considerably lower then the incidence detected in shrimp for the same time period (for crabs: late May = 2.5% and early June = 3.3%) (Fig. 3a). The percentage of crab stomachs testing positive decreased to 0.0% by mid-June. In contrast to shrimp, however, 7.3% of the gut material from crabs collected between early July and August tested positive for juvenile flounder; hence, sampling date was not a significant factor in the proportion of crab stomachs testing positive for juvenile flounder remains (Fig. 3a; Table 4).

The mean size of post-settlement flounder in Niantic River, Connecticut, sampled in late May was 12.5 mm TL (Fig. 3b). Flounder body size then increased at a decelerating rate during the summer such that the mean length of juveniles in early August was 28.4 mm TL (Fig. 3b).

4. Discussion

Predation on the early life history of fish is an important factor regulating year-class formation and recruitment. However, quantifying predatory impacts is particularly difficult when analyses rely on visually identifying the remnants of partially digested fish in the stomachs of suspected predators. The direct observation of decapod crustacean diets is especially challenging because mandible and gastric mill grinding renders fish tissue visually unidentifiable. Moreover, determining in situ predation rates by identifying fish hard parts in predator stomachs may lead to erroneous conclusions because of crustaceans ingesting particulate matter and other prey items that visually resemble the target prey, and incomplete or partial predation events that do not result in the ingestion of identifiable fish remains (Feller, 1991; Seikai et al., 1993). Thus, previous studies examining the trophic dynamic role of crustaceans may need to be reevaluated if conclusions were derived from visual dietary analysis.

The immunoassay described in this study offers an alternative approach to visual estimations by providing a means of positively identifying flounder proteins in the stomach contents of shrimp and crab predators. Other biochemical methods, including DNA-based assays (Rosel and Kocher, 2002) and variations of the prescribed serological techniques (Theilacker et al., 1986; Hentschel and Feller, 1990; Schultz and Clarke, 1995), have successfully analyzed the diet of different organisms feeding on the early life stages of marine fish. The immunodiffusion method has several advantages relative to other biochemical techniques, including that the procedure is fundamentally simple, methodological constraints are minimal, and large quantities of samples can be processed quickly and inexpensively without sacrificing detection sensitivity (Feller, 1992). The type of immunoassay used in this study is limited, however, in that it only provides qualitative responses, i.e., the presence or absence of a particular prey item in the predator's stomach. Alternative immunological techniques quantify protein concentrations (e.g., rocket immu-

noelectrophoresis: Hentschel and Feller, 1990; Feller, 1991), but these results are not necessarily interpretable in an ecologically meaningful way (Feller, 1991). Quantitative immunoassays, for example, cannot distinguish among the causative factors resulting in the amount of prey in a predator's stomach, such as the meal size consumed at a discrete feeding event, multiple feeding events on the same species, and the time elapsed between feeding and the analysis of gut contents.

All immunoassays are limited by the quality of the antisera used to detect target prey, whereby errors in analysis are caused by false-negative or false-positive results. The failure of an assay to detect target prey that are present in a predator's stomach (false-negativity) can be caused by insensitive antisera or testing beyond detection limits. Detection limits are mostly dependent on predator digestion rates, which in turn are influenced by a variety of external conditions (e.g., temperature, meal composition and size). Of greater concern is the incorrect assertion that a predator has consumed a target prey, or a false-positive result. This is frequently caused by nonspecific antiserum that cross-reacts with heterologous antigens from the predator or nontarget prey. Antibody specificity can be magnified substantially through the use of monoclonal antibodies, but monospecific antisera are fundamentally limited in other ways that diminish their utility (Feller, 1991). For example, a monoclonal antibody may recognize a very specific complementary antigenic determinant located on a target protein. However, the integrity of this specific binding site could be destroyed during digestion, thus giving the antiserum low sensitivity in detecting target prey. Monospecific antisera may also react with specific binding sites that exist on a variety of very different antigens rather than on a single target protein of interest (Feller, 1991). Protein structures are radically altered during digestion and reveal binding sites that may not have been present in the protein's native form. Consequently, monospecific antisera might react with portions of proteins completely unrelated to the target prey (Feller, 1991). False-positive results may also be caused by circumstantial limitations and not methodological problems with antisera, such as predators scavenging on remnants of target prey, predation in nets during field sampling, and secondary detection, i.e., stomach contents of a predator contain prey whose stomach contents contain the prey of interest (Feller, 1992).

Winter flounder-specific antisera used in this study successfully identified homologous antigens without appreciably cross-reacting with antigenic material from predator or nontarget prey. Flounder eggs and juveniles were also detected in predator stomach samples following ingestion of prey in laboratory feeding experiments. However, the ability of antisera to identify target prey in stomach samples was considerably lower relative to reactions observed with pure homologous extracts. The decrease in detection capability was attributed to the mastication of prey tissue by crustacean mandible and gastric mill grinding that radically altered protein structures and denatured antibody–antigen binding sites present in the protein's native form. Digestion continued to decrease the detection strength of target prey in predator stomachs. The detection limit for flounder eggs occurred 10.8–16.4 h after initial ingestion by various sized shrimp, and juvenile flounder were detectable 9.4 and 7.8 h post-feeding for shrimp and crabs, respectively. The longer detection time of eggs in shrimp stomach samples, relative to juveniles, was attributed to colder temperatures during egg feeding experiments, which in turn decreased predator digestion and evacuation rates (Van der Veer and Bergman, 1987).

Detection limits in this study were defined by the formation of at least one distinct precipitin line when flounder-specific antisera were tested against solubilized predator gut material. Previous investigators have implemented more conservative methods for defining positive identification of target prey (Hunter and Feller, 1987; Van der Veer et al., 1998). For example, algorithms derived from the direct comparison of self- and cross-reactions are frequently used to ascertain whether the presence of a particular prey is actual or artificial due to a false-positive reading (Hunter and Feller, 1987; Feller, 1991). Such stringent controls were relaxed in this study for several reasons. The level of cross-reaction between flounder-specific antisera and antigenic material from predators and nontarget prey was absent or minimal. Moreover, the number of precipitin lines formed when antisera are crossed with pure heterologous antigens will decrease substantially after nontarget prey are ingested by crustaceans. For example, the strongest level of cross-reaction was observed when juvenile flounder antiserum was tested against northern kingfish (*Menticirrhus saxatilis*). Accordingly, an average of 4.0 precipitin lines were formed in this cross-reaction, which represents a 71% decrease in detection strength relative to self-reactions with pure homologous extracts. Assuming that the recognition of digested *M. saxatilis* decreases by 52% for shrimp and 74% for crabs following initial feeding (as observed with flounder tissue), the expected number of precipitin lines formed immediately after ingestion is 1.9 lines for shrimp and 1.0 lines for crabs. Subsequent to the decrease in response strength with predator digestion, the detection limit of *M. saxatilis* in shrimp and crab stomachs is projected to occur at 0.6 and 0.0 h after initial feeding. Suspected predators of winter flounder eggs and juveniles were collected from the field during daylight hours. Crustaceans are nocturnal predators (Wilcox and Jeffries, 1974; Pihl and Rosenberg, 1984; Ansell and Gibson, 1993), and therefore it is unlikely that the ingestion of nontarget prey during the previous night would be detected with the immunoassay. Conversely, the detection limit of homologous antigens in crustacean diets (eggs: 10.8–16.4 h post-feeding; juveniles: 7.8–9.4 h post-feeding) is sufficiently long to identify target prey in predator gut contents even if the consumption of prey occurred many hours prior to dietary analysis.

This study provides the first evidence of *C. septemspinosa* and *C. maenas* predation on the early life stages of winter flounder in natural populations. The presence of winter flounder eggs and juveniles in *C. septemspinosa* stomach samples suggested a temporal trend of decreasing predation pressure during winter and summer field surveys, respectively. The incidence of eggs in shrimp stomachs, for example, was maximal in mid-February at the height of the winter flounder spawning period. Predation on flounder eggs then decreased significantly over time, reflecting the end of the spawning season and the relatively low abundance of eggs during early spring.

C. septemspinosa predation on post-settlement flounder was maximal in late May and significantly decreased at a decelerating rate over time. The probable cause for this temporal trend is shrimp size-dependent predation on newly settled flatfish (Van der Veer and Bergman, 1987; Witting and Able, 1995; Taylor, in press). Winter flounder are most susceptible to *C. septemspinosa* predation at settlement, when fish are small in size and completing several aspects of morphological and ecological metamorphosis (Witting and Able, 1995; Taylor, in press). Susceptibility to shrimp predation then gradually decreases after settlement until a refuge is attained when flounder reach a size of 25 mm TL (Witting

and Able, 1995; Taylor, in press). In late spring and early summer, the average body size of post-settlement flounder in Niantic River, Connecticut, was 12.5 and 21.1 mm TL, respectively; hence at the onset of field sampling juvenile flounder were vulnerable to *C. septemspinosa* predation. By mid-June, however, juveniles reached an average size of 25.2 mm TL, which then gradually increased during the summer and precluded the flatfish as prey for shrimp. The seasonal pattern of flounder growth and body size is the probable mechanism underlying the observed incidence of juveniles in the stomach contents of *C. septemspinosa*. Similar conclusions were made in the western Wadden Sea, whereby *C. crangon* predation on juvenile plaice (*P. platessa*) was restricted to the period between initial plaice settlement and late May and early June, when plaice populations achieved a mean length of approximately 30 mm (Zijlstra et al., 1982; Van der Veer and Bergman, 1987).

C. maenas predation on newly settled flatfish is also governed by size-dependent relationships between predator and prey (Van der Veer and Bergman, 1987; Fairchild and Howell, 2000). Fairchild and Howell (2000), for example, demonstrated that larger crabs have a greater capacity to capture, subdue, and consume juvenile winter flounder. Moreover, large *C. maenas* (>30 mm CW) were capable of feeding on flounder of a body size of 70 mm TL (Fairchild and Howell, 2000). Van der Veer and Bergman (1987) also observed that *C. maenas* (>26 mm CW) successfully preyed on juvenile *P. platessa* up to 50 mm in total length. In this study, there was no consistent temporal trend in *C. maenas* predation on post-settlement flounder during late spring and summer. In contrast to the prey size constraints imposed on *C. septemspinosa*, juvenile flounder did not attain a size refuge from crab predation during the sampling period (maximum size of flounder equaled 28.4 mm TL in early August). The insignificant relationship between sampling date and the proportion of *C. maenas* stomachs containing flounder was attributed to prey remaining vulnerable to crab predation during the entire summer. In summary, the temporal trends in post-settlement flounder mortality reflect the size-dependent relationships specific to the given crustacean and flounder predator–prey interaction.

The primary contribution of this research is the presentation of an immunochemical technique that provides a means of identifying the proteins of early-stage fish in the stomach contents of suspected predators. In the process, the diet of field-collected crustaceans was examined, and the first tangible evidence is given indicating that *C. septemspinosa* and *C. maenas* are predators on the early life stages of winter flounder in natural populations. The cumulative effect of shrimp and crabs on flounder egg and juvenile mortality is beyond the scope of this work. To effectively address the role of decapod crustaceans in governing flounder year-class strength and recruitment, future research must address: (1) the spatial and temporal overlap between crustacean predators and the specific life stage of winter flounder in question, (2) the quantification of predator and prey densities through comprehensive field surveys, and (3) the potential effects of biotic and physical factors (e.g., density, temperature) on predator and prey behavior and physiology.

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References

Ansell, A.D., Gibson, R.N., 1993. The effect of sand and light on predation of juvenile plaice (*Pleuronectes platessa*) by fishes and crustaceans. J. Fish Biol. 43, 837–845.

Bailey, K.M., Houde, E.D., 1989. Predation on eggs and larvae of marine fishes and the recruitment problem. Adv. Mar. Biol. 25, 1–83.

Bailey, K.M., Brodeur, R.D., Merati, N., Yoklavich, M.M., 1993. Predation on walleye pollock (*Theragra chalcogramma*) eggs and yolk-sac larvae by pelagic crustacean invertebrates in the western Gulf of Alaska. Fish. Oceanogr. 2, 30–39.

Chambers, R.C., Leggett, W.C., 1987. Size and age at metamorphosis in marine fishes: an analysis of laboratory-reared winter flounder (*Pseudopleuronectes americanus*) with a review of variation in other species. Can. J. Fish. Aquat. Sci. 44, 1–15.

Day, R.W., Quinn, G.P., 1989. Comparisons of treatments after an analysis of variance in ecology. Ecol. Monogr. 59, 433–463.

Edwards, R., Steele, J., 1968. The ecology of 0-group plaice and common dab at Loch Ewe: I. Population and food. J. Exp. Mar. Biol. Ecol. 2, 215–238.

Fairchild, E.A., Howell, W.H., 2000. Predator–prey size relationship between *Pseudopleuronectes americanus* and *Carcinus maenas*. J. Sea Res. 44, 81–90.

Feller, R.J., 1991. Dietary analysis of penaeid shrimp: the immunoassay approach. In: DeLoach, P., Dougherty, W.J., Davidson, M.A. (Eds.), Frontiers of Shrimp Research. Elsevier, Amsterdam, pp. 141–156.

Feller, R.J., 1992. Potential applications of immunoassays in studies of flatfish recruitment. Neth. J. Sea Res. 29, 1–5.

Feller, R.J., Taghon, G.L., Gallagher, E.D., Kenny, G.E., Jumars, P.A., 1979. Immunological methods for food web analysis in a soft-bottom benthic community. Mar. Biol. 54, 61–74.

Frank, K.T., Leggett, W.C., 1984. Selective exploitation of capelin (*Mallotus villosus*) eggs by winter flounder (*Pseudopleuronectes americanus*): capelin egg mortality rates and contribution of egg energy to the annual growth of flounder. Can. J. Fish. Aquat. Sci. 41, 1102–1294.

Haegele, C.W., 1993a. Seabird predation of Pacific Herring, *Clupea pallasi*, spawn in British Columbia. Can. Field-Nat. 107, 73–82.

Haegele, C.W., 1993b. Epibenthic invertebrate predation of Pacific Herring, *Clupea pallasi*, spawn in British Columbia. Can. Field-Nat. 107, 83–91.

Hentschel, B.T., Feller, R.J., 1990. Quantitative immunoassay of the proventricular contents of white shrimp, *Penaeus setiferus* Linnaeus: a laboratory study. J. Exp. Mar. Biol. Ecol. 139, 85–99.

Hossain, M.A.R., Tanaka, M., Masuda, R., 2002. Predator–prey interaction between hatchery-reared Japanese flounder juvenile, *Paralichthys olivaceus*, and sandy shore crab, *Matuta lunaris*: daily rhythms, anti-predator conditioning and starvation. J. Exp. Mar. Biol. Ecol. 267, 1–14.

Hunter, J., Feller, R.J., 1987. Immunological dietary analysis of two penaeid shrimp species from a South Carolina tidal creek. J. Exp. Mar. Biol. Ecol. 107, 61–70.

Kellison, G.T., Eggleston, D.B., Burke, J.S., 2000. Comparative behaviour and survival of hatchery reared versus wild summer flounder (*Paralichthys dentatus*). Can. J. Fish. Aquat. Sci. 57, 1870–1877.

Klein-MacPhee, G., Howell, W.H., Beck, A.D., 1982. Comparison of a reference strain and four geographical

strains of *Artemia* as food for winter flounder, *Pseudopleuronectes americanus* larvae. Aquaculture 29, 279–284.

Lockwood, S.J., 1980. Density-dependent mortality in 0-group plaice (*Pleuronectes platessa* L.) populations. J. Cons.-Cons. Int. Explor. Mer 39, 148–152.

McTigue, T.A., Feller, R.J., 1989. Feeding of juvenile white shrimp *Penaeus setiferus*: periodic or continuous? Mar. Ecol. Prog. Ser. 52, 227–233.

Pearcy, W.G., 1962. Ecology of an estuarine population of winter flounder, *Pseudopleuronectes americanus* (Walbaum): Parts I–IV. Bull. Bingham Oceanogr. Collect. 18, 1–78.

Pihl, L., Rosenberg, R., 1984. Food selection and consumption of the shrimp *Crangon crangon* in some shallow areas in western Sweden. Mar. Ecol. Prog. Ser. 15, 159–168.

Rogers, C.A., 1976. Effects of temperature and salinity on the survival of winter flounder embryos. Fish. Bull. US 74, 52–58.

Rosel, P.E., Kocher, T.D., 2002. DNA-based identification of larval cod in stomach contents of predatory fishes. J. Exp. Mar. Biol. Ecol. 267, 75–88.

Schultz, D.R., Clarke, M.E., 1995. An immunological study of predation on hatchery-reared, juvenile red drum (*Sciaenops ocellatus*): preparation and assays of a "red drum-specific" protein for predator–prey experiments. J. Exp. Mar. Biol. Ecol. 189, 233–249.

Scott, W.B., Scott, M.G., 1988. Atlantic fishes of Canada. Can. Bull. Fish. Aquati. Sci. 219, 731.

Seikai, T., Kinoshita, I., Tanaka, M., 1993. Predation by crangonid shrimp on juvenile Japanese flounder under laboratory conditions. Nippon Suisan Gakkaishi 59, 321–326.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., Klenk, D.C., 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76–85.

Taylor, F.H.C., 1964. Life history and present status of British Columbia herring stocks. J. Fish. Res. Board Can. 143 (81 pp.).

Taylor, D.L., in press. Size-dependent predation on post-settlement winter flounder (*Pseudopleuronectes americanus*) by sand shrimp (*Crangon septemspinosa*). Mar. Ecol. Prog. Ser.

Theilacker, G.H., Kimball, A.S., Trimmer, J.S., 1986. Use of an ELISPOT immunoassay to detect euphausiid predation on larval anchovy. Mar. Ecol. Prog. Ser. 30, 127–131.

Theilacker, G.H., Lo, N.C.H., Townsend, A.W., 1993. An immunochemical approach to quantifying predation by euphausiids on the early stages of anchovy. Mar. Ecol. Prog. Ser. 92, 35–50.

Underwood, A.J., 1981. Techniques of analysis of variance in experimental marine biology and ecology. Oceanogr. Mar. Biol. Ann. Rev. 19, 513–605.

Van der Veer, H.W., Bergman, M.J.N., 1987. Predation by crustaceans on newly settled 0-group plaice *Pleuronectes platessa* populations in the western Wadden Sea. Mar. Ecol. Prog. Ser. 35, 203–215.

Van der Veer, H.W., Feller, R.J., Weber, A., Witte, J.I.J., 1998. Importance of predation by crustaceans upon bivalve sprat in the intertidal zone of the Dutch Wadden Sea as revealed by immunological assays of gut contents. J. Exp. Mar. Biol. Ecol. 231, 139–157.

Wilcox, J.R., Jeffries, H.P., 1974. Feeding habits of the sand shrimp *Crangon septemspinosa*. Biol. Bull. 146, 424–434.

Witting, D.A., Able, K.W., 1995. Predation by sevenspine bay shrimp *Crangon septemspinosa* on winter flounder *Pleuronectes americanus* during settlement. Mar. Ecol. Prog. Ser. 123, 23–31.

Yamashita, Y., Yamada, H., Malloy, K.D., Targett, T.E., Tsuruta, Y., 1996. Sand shrimp predation on settling and newly-settled stone flounder and its relationship to optimal nursery habitat selection in Sendai Bay, Japan. Proceedings of an International Workshop: Survival Strategies in Early Life Stages of Marine Resources. Balkema, Rotterdam, pp. 271–284.

Zijlstra, J.J., Dapper, R., Witte, J.I.J., 1982. Settlement, growth and mortality of post-larval plaice (*Pleuronectes platessa*) in the Western Wadden Sea. Neth. J. Sea Res. 15, 250–272.